PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
B01J 20/32, B01D 15/08 G01N 30/48, 33/543	A1	WO 94/11103
		(43) International Publication Date: 26 May 1994 (26.05.94)

(21) International Application Number: PCT/GB93/02289

(22) International Filing Date: 5 November 1993 (05.11.93)

(30) Priority data:
9223334.5 6 November 1992 (06.11.92) GB

(71) Applicant *(for all designated States except US)*: WILLIAMS, John, Francis [GB/GB]; 4 Cranmer Road, Hampton, Middlesex TW12 1DW (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : BRUCE, Ian, James [GB/GB]; 90 Lower Park Road, Hastings, East Sussex TN34 2LE (GB). DAVIES, Martin, John [GB/GB]; 27 West Hill, Wandsworth, London SW18 1RB (GB). SCOPES, Geoffrey, Eric [GB/GB]; 18 Ingram House, Park Road, Kingston, Surrey KT1 4BA (GB). SMETHURST, Diane, Elizabeth [GB/GB]; 83 The Grove, North Cray, Sidcup, Kent DA14 5NG (GB).

(74) Agents: WILLIAMS, John, Francis et al.; Williams, Powell & Associates, 34 Tavistock Street, London WC2E 7PB (GB).

(81) Designated States: AU, CA, GB, JP, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: MAGNETIC SOLID PHASE SUPPORTS

(57) Abstract

Biomolecules are separated using magnetisable polymer-based particles derivatised with a ligand having direct binding affinity for nucleic acids or other types of biopolymer molecule. The particles are used for nucleic acid (and other biomolecular) purification and speciations, including double or single stranded nucleic acids, genomic DNA, plasmid DNA, and cellular RNA.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

MAGNETIC SOLID PHASE SUPPORTS

This invention relates to the isolation and purification of molecules of biological interest eg proteins, nucleic acids, and other biopolymers.

The purification of molecules of the above kinds often involve multi-step procedures requiring much time, technically complex equipment and potentially hazardous reagents. An affinity chromatography step is often the final or penultimate step. Ideally this step should be employed as early as possible in the process to maximise the yield of desired product and minimise the time required to generate it. Usually this is not feasible as the substance of interest (ligate) is present in a medium not readily amenable to conventional column affinity chromatography. Often the ligate may be present in a mixture of substances containing particulate and/or semi-solid or colloidal material such as cell debris and denatured cell components and/or in a mixture which is highly viscous. Column chromatography demands that both these factors be as low as possible and the steps in a purification procedure prior to affinity chromatography are engaged in minimising these two factors. They also result in the loss of at least some of the desired end product and an increase in the time required to collect it.

The present invention is concerned with the development of relatively simple techniques for use with complex mixtures of high viscosity and/or those containing particulate matter and which are based on the use of magnetisable solid phase support (MSPS) materials. The use of magnetic separation in biological systems has been proposed in recent years. However, in spite of the

fact that the facilities required for magnetic separation are much less costly and complex than those required for column chromatography or centrifugation the techniques have not so far received the attention they deserve.

The present invention comprises magnetisable polymer based particles derivatised with ligands possessing direct-binding affinities for nucleic acids and other types of biomolecule, and the use of these particles for nucleic acid (and other biomolecular) purification and speciation, including double or single stranded nucleic acids, genomic DNA, plasmid DNA, and cellular RNA.

The direct binding of the ligand to the target molecule is important to achieve the benefits of this invention as contrasted with prior proposals based on indirect linking of the target molecules eg. through intercalating agents as described in EP 301,899 A.

The present invention comprises particulate support material having magnetic properties and bearing a ligand which binds to molecules of a specific type. From another aspect the present invention comprises a method of separating biomolecules of various types by binding such molecules to ligands which are selective therefore and which are attached to magnetisable particulate support materials. The invention is further apparent from the appended claims.

The magnetic susceptibility of MSPS materials in accordance with the present invention is a function of the weight and size of the particulate material and the quantity of paramagnetic material incorporated therein. This property may therefore be adjusted as desired in order to provide an appropriate selection parameter. In

practice a product range of MSPS materials may be manufactured in order to cater for the wide range of separations encountered in both analytical and preparative procedures.

The MSPS materials in accordance with this invention are preferably gels and are conveniently in the form of beads. Such gels may be stabilised by crosslinking eg with epichlorohydrin, leading to greater particle stability especially at high temperatures. Many polymers may be used for the purposes of this invention including cellulose, dextran, polyacrylamide and the synthetic material trisacryl. One of the most preferred materials is agarose. The positive benefits of this polymer are gel strength and biological inertness. The abundance of hydroxyl groups possessed by the agarose matrix allows for easy covalent attachment of various ligands desirable for molecular biology applications and its non-covalent secondary structure also engenders structural stability, porosity and ease of shaping into spheres. Since many molecular biology methodologies are performed at temperatures above that at which agarose melts it would also be desirable to introduce thermal stability into the agarose solid phase support.

MSPS materials consist of at least two separate components a non-magnetisable component which may be chemically derivatised with suitable ligands and a magnetisable component or core. Paramagnetic properties can be introduced into the solid phase support by addition of powdered paramagnetic iron oxide (Fe_3O_4) to the agarose during the preparation of the MSPS, which leads to entrapment of the paramagnetic component within the solid phase matrix and gives the particles their magnetisability.

MSPS particles should be within a size range which allows the greatest particle surface area exposure to ligate, but at the same time allows the best particle magnetic sedimentation rate. A relationship exists between spherical particle volume and sedimentation rate controlled by the equation $F = (X_V - X_V^O)\ VH\ (dH/dX)$ where F = net force X_V and x_V^O = the volume susceptibilities, and V = the volume of the particle and H = applied magnetic field and dH/dX + the magnetic field gradient. The magnetisable component should also have a high degree of magnetisable susceptibility to optimise magnetic sedimentation rates.

The ideal magnetisable component can be selected on the basis of the above equation, but in practice relatively few paramagnetic molecules have been used in MSPS. Iron oxide, barium ferrite and nickel oxide may be used. Iron oxide has many advantages over other substances for MSPS construction since it possesses a high magnetic susceptibility, is readily available, inexpensive and non toxic. A highly preferred system for our purposes entails iron oxide as the magnetisable component for MSPS and agarose as the polymer matrix in which to embed it. However, other natural and synthetic polymers may be used as MSPS matrix material including cellulose, alginate, dextran and perfluorocarbon based supports.

MSPS may be prepared either by encapsulation of the magnetisable component during the preparation of the particle, or by addition of the magnetisable component subsequent to particle formation.

Encapsulation

Encapsulation of the magnetisable component is convenient when iron oxide is used in MSPS construction.

The magnetisable component can be mixed in an aqueous suspension of cellulose or agarose and allowed to cure, the water is removed and the dried material ground to the size range required.

Alternatively, MSPS may be prepared by spraying or droplet formation and this leads to beaded particles. MSPS may be prepared by addition of a suspension of sodium alginate and iron oxide dropped into a solution of calcium chloride. Droplet formation can be brought about through emulsification techniques and thee have been employed for the formation of spherical magnetisable dextran, albumin, acrylates, acrolein and polyglutaraldehyde. In this system an aqueous emulsion of the matrix polymer and magnetisable component is prepared. This is added to mineral oil and the two phase system stirred. A precipitate is formed of beaded particles.

Subsequent addition

Preparation of MSPS by addition of the magnetisable component after particle formation has been achieved with derivatised and underivatised sepharose. It has been reported that coating sepharose particles with a suspension of ferrite in water or hydrocarbon solvent results in their magnetisability without significant loss of activity of the derivatised form.

As an alternative to this method the particle is coated with an aqueous solution of polyacrylamide containing ferric oxide. The resulting particles tend to be large >250 μm and very porous. However, they are amenable to activation via either the polysaccharide or polyacrylamide component.

Preparation of MSPS

In one method we have used to prepare agarose MSPS an aqueous suspension is extruded into an immiscible organic phase. A suspension of paramagnetic iron oxide, Fe_3O_4, (4% w/v) in molten agarose (2% w/v) was extruded into vegetable oil stirred with an overhead paddle stirrer using a specially adapted plastic syringe. The end of the syringe was sealed, then drilled with a small hole to allow the molten agarose/iron oxide mixture to be extruded into the oil phase. The MSPS was collected, washed with water and sized by sieving, then checked under a light microscope set up for Köhler illumination for morphological homogeneity. The particles formed were highly bead like and uniformly spherical, a consequence of both the extrusion technique and the immiscibility of the oil and water.

The highly spherical nature of the particles produced is particularly desirable because it prevents local concentration change effects that might occur with particles whose surface is more heterogeneous. It is particularly important to maintain uniform ionic concentrations around the surface of a particle in order that local absorption and/or desorption does not occur and alter the bulk properties of the support.

When the MSPS was viewed under the light microscope, small yellow inclusions were observed incorporated within the agarose matrix which were believed to be vegetable oil retained in the matrix of the agarose during the preparation of the MSPS. To remove these inclusions the MSPS was washed following preparation in an acetone/water series.

Many factors determine the size, quantity and quality of

the MSPS produced. A range of differently sized MSPS (>500μm - <150μm) were obtained when stir rate, hole bore diameter and extrusion rate were altered. Results indicated that the faster the stir rate of the oil phase, the narrower the bore size of the pierced hole and the higher the extrusion rate, the greater the proportion of smaller diameter MSPS (less than 150μm) was produced. Small particles are sought for affinity chromatography as they possess a greater surface area/volume ratio, significantly enhancing their ligand loading capacity and subsequent ability to take up and release macromolecules during chromatography.

In an illustrative preparation, 10 ml of molten agarose/iron oxide suspension was extruded from a 10ml syringe at a constant extrusion rate of 3.3 ml sec^{-1} (10ml extruded over a period of 3 sec) into vegetable oil stirred at ~6000 rpm. The effect of bore size diameter on MSPS production was studied first, with holes of 0.7, 0.5, 0.3 and 0.1mm being drilled in the sealed end of the syringe. A diameter of 0.7mm gave the largest amount of MSPS greater than 500μm diameter, whilst negligible amounts of the smaller sized particles (less than 150μm diameter) were formed. Using syringes drilled with 0.5mm and 0.3mm diameter holes gave a slight increase of these smaller sized MSPS, but large amounts of the particles greater than 500μm were still produced. Only when a syringe with a hole bore size of 0.1mm was used was there a significant increase in the proportion of smaller diameter MSPS produced.

The effect of different extrusion rate on MSPS production, has also been investigated using a syringe of drilled hole bore of 0.1mm and stir rate of 6000rpm. The greater the extrusion rate, the greater the proportion of smaller diameter MSPS collected, and a

rate of 3.3 ml sec^{-1} (rate measured as above) gave the best results. Similarly, we have found that increasing the stir rate leads to an increase in the amount of smaller sized (less than 150μm diameter) MSPS produced.

Taken together, the above results indicate that the conditions which favour production of smaller MSPS are those of small hole bore size, high extrusion rate and stir rate. Although the size range distribution of the agarose MSPS produced is wide, sufficient amounts of small particles can be produced for derivatisation and applications. It was observed that 20μm appeared to be the minimum diameter of MSPS attainable using this production method, any material recovered below this size generally consisted of non-spherical lumps and waste material of varying iron oxide/agarose content.

In order to confer thermal and chemical stability on the MSPS following manufacture, it may be cross-linked with epichlorohydrin under basic conditions using known methods. The cross-linking reaction covalently binds together the polymeric agarose strands by a three-carbon link, instilling a physical rigidity in the particles that reduces the risk of shear damage. Once cross-linked MSPS can be autoclaved (120°C, 15psi, 15 min) without damage or alteration of its physical properties, and is resistant to a whole range of chemical reagents used in further derivatisations. Cross-linked MSPS can be stored quite satisfactorily at room temperature for several months as a suspension in 20% aqueous methanol (to act as an anti-bacterial agent) without any deterioration. No leaching of iron oxide from the agarose matrix was observed.

Surface derivatisation of MSPS with affinity ligands

Many procedures already exist for the derivatisation of cellulose, dextran and agarose with affinity ligands, and these can be utilised with a minimum of adaption for our agarose MSPS.

We have prepared several MSPS derivatives for nucleic acid purification. These are listed below; the type of surface ligand is given along with its abbreviated form:

(i) Diethylaminoethyl - DEAE
(ii) Epichlorohydrin/triethanolamine - ECTEOLA
(iii) Spermine
(iv) Diglycidylbutane-1,4,-diol/Tris(aminoethyl) amino - TAEA
(v) Epichlorohydrin/hydroxyethylpiperazine - EHEP
(vi) Diglycidylbutane-1,4,-diol/Hexane-1,6-diamino - HDA

DEAE-, ECTEOLA-, spermine-, TAEA-, EHEP, and HDA-MSPS can all be used for the general isolation of nucleic aids and are listed in decreasing order of affinity for nucleic acids.

The degree of ligand loading may be expressed as the ability of a known weight of MSPS to adsorb a known amount of DNA, and the adsorption measured by ultra-violet spectroscopy. This serves as a method of quality control for the MSPS to check that surface derivatisation had proceeded satifactorily. In an illustrative procedure, a suspension of DEAE-MSPS (100 mg ml^{-1}) was incubated with a solution of salmon sperm DNA (50μg ml^{-1}) at room temperature in an Eppendorf tube. After 30 minutes the MSPS was immobilised with a magnetic concentrator, the supernatant removed and its adsorbance at 260nm and 280nm recorded, and compared with that of the original stock solution of DNA to

calculate the level of nucleic acid uptake. Typically, for DEAE-MSPS a figure of at least 95% uptake of salmon sperm DNA was obtained. Similar results were obtained for ECTEOLA-, TAEA- and spermine-MSPS (Table 1).

The nitrogen atoms of the ligand become protonated in solution, generating a positively charged MSPS to which the negatively charged phosphate backbone of the nucleic acid is attracted (Fig. 1). Since tertiary amines are more basic than secondary or primary amines, solid phase supports derivatised with tertiary amine-containing ligand have a higher positive charge density and therefore a greater affinity for nucleic acids. This has been confirmed in so far as the DEAE, ECTEOLA and TAEA supports performed best in the DNA uptake assay, whilst the EHEP, and HDA-MSPS showed decreasing affinity for DNA , not because of a low degree of surface ligand coverage (the amounts of reagents used were sufficient to ensure high surface derivatization), but because of the lower affinity of the secondary and primary amine ligands for DNA. The spermine-MSPS displayed good affinity for DNA possibly because its long chain polyamine nature enables it to bind DNA molecules by wrapping around them and that the ionic charge of attraction of the multi-protonated polyamines is greater.

The adsorbed DNA could be eluted from all samples with at least 80% efficiency (based on A_{260} readings) by incubation for up to 30 minutes with 1M NaCl/50mM arginine free base at 65°C. The derivatised MSPS can be stored at 4°C for several months in suspensions of 20% aqueous methanol without any decrease in performance or capacity for nucleic acids.

EXAMPLE 1 Preparation of beaded magnetic agarose

Two methods for the preparation of magnetisable solid phase support (MSPS) are given below.

(i) A solution of molten agarose (2% w/v) containing paramagnetic iron oxide, Fe_3O_4 (4% w/v), and sodium azide (0.02% w/v) is extruded at a rate of 3.3 ml s^{-1} from a 0.1 mm diameter hole drilled at the end of a sealed 10ml syringe into vegetable oil (100ml), rapidly stirred by an overhead paddle stirrer at 6000 rpm. Stirring is continued for 1 minute after extrusion is complete then 100ml of deionised water is added and the two-phase mixture left to stand on a slab magnet for 16 hours. The majority of the cleared oil phase is decanted and the aqueous phase containing the beaded MSPS re-washed with deionised water (100ml). The suspension of MSPS is initially sized by sieving through a series of Endecott sieves of mesh size 500μm, 250μm, 200μm, 180μm, and 150μm using a Fritsch sieve shaker. The fraction containing particles of less than 150μm diameter is further sieved with sieves of mesh size 100μm, 50μm, 32μm and 20μm. All fractions collected are then washed successively with 30:70 v/v acetone:water, 70:30 v/v acetone:water then 100% anhydrous acetone, and the MSPS is stored in 20% aqueous methanol to act as an anti-bacterial agent.

(ii) The procedure of (1) is followed except that homogenisation is used instead of overhead stirring. The initial solution is extruded into vegetable oil stirred at 11,000 rpm in a homogeniser. Homogenisation is continued for a further 1 minute after extrusion is complete then 100 ml of deionised water is added and the two-phase mixture left to stand on a slab magnet for 16 hours. The remaining procedure is as described in (1).

Particles of diameter 20-50μm or 50-100μm are selected for further derivatization with affinity ligands and biological applications. and the greatest proportion of these sizes is produced using the homogeniser method.

Thermal stability is introduced into the MSPS prepared as described above by crosslinking agarose polymers using epichlorohydrin. Two methods have been used:

1. 10ml of settled volume of MSPS is stirred with 100ml 0.05 M NaOH and 1ml epichlorohydrin using a rotary shaker at room temperature for 3 h. Subsequently, the MSPS is washed several times with 0.05M NaOH.

2. 10 ml of settled volume MSPS is stirred with 100 ml 0.05 M NaOH, 1 ml epichlorohydrin and 0.05g $NaBH_4$ using a rotary shaker for 2 h at 60°c. Subsequently, the MSPS is washed several times with warm distilled water.

EXAMPLE 2 Preparation of derivatised MSPS

(i) DEAE

Cross-linked MSPS (3.2 g moist weight) is suspended in 10% sodium hydroxide (15ml) and cooled to 0°C in an ice-water bath. A solution of 2-(N-N'-diethylamino)ethyl chloride hydrochloride (3g) in water (4ml) is added slowly to the suspension with occasional shaking. The suspension is left at 0°C for 30 minutes, then allowed to warm 23°C. After 16 hours, the DEAE-MSPS is washed successively with 2M sodium chloride, sterile distilled water, 30:70 v/v ethanol:water then 70:30 v/v ethanol:water, and finally suspended in 20% aqueous methanol to give a final particle density of 100 mg/ml.

(ii) ECTEOLA

Cross-linked MSPS (0.7 g moist weight) is suspended in 40% sodium hydroxide (3ml) and cooled to 0°C in an ice-water bath. A solution of triethanolamine (0.4ml) in epichlorohydrin (0.7ml) is added drop-wise with occasional shaking, maintaining the temperature at 0°C. After 30 minutes the suspension is allowed to warm 23°C, shaken vigorously using a flask shaker and left to stand for 16 hours. The mixture is then poured into a large volume of 1M hydrochloric acid with stirring, filtered, then washed successively with 1M sodium hydroxide, sterile distilled water, 20% aqueous methanol then finally water. The ECTEOLA-MSPS is stored in 20% aqueous methanol to give a particle density of 100 mg/ml.

(iii) Spermine

Cross-linked MSPS (0.5 g moist weight) is washed successively with 30:70 v/v dioxane:water, 70:30 v/v dioxane:water, anhydrous dioxane and finally suspended in anhydrous dioxane (1ml). 1,1-carbonyldiimidazole (0.1g) is added under a nitrogen atmosphere and the suspension shaken for 30 minutes at 23°C. The MSPS is washed again with anhydrous dioxane, the cooled to 0°C. A solution of spermine (0.56g) in deionised water (2.5ml) is slowly added with agitation, and the suspension of MSPS left at 4°C for 16 hours. It is then shaken for a further 2 hours at 23°C, then washed successively with sterile distilled water, 1M hydrochloric acid and finally sterile distilled water. The spermine-MSPS is stored in 20% aqueous methanol to a final particle density of 100 mg/ml.

(IV) TAEA

Cross-linked MSPS (0.5 g moist weight) is suspended in 1M sodium hydroxide (0.4ml). Sodium borohydride (1mg) is added followed by diglycidylbutane-1,4-diol (0.5ml). The suspension is shaken at 23°C for 6 hours, then filtered, washed thoroughly with deionised water and immediately resuspended in 5M tris(2-aminoethyl)amine (1ml) and shaken at 30°C for 2 hours. The suspension is washed successively with sterile distilled water, 1M sodium chloride, sterile distilled water and finally 0.05M phosphate buffer (pH7). The TAEA-MSPS is stored in 20% aqueous methanol to a final particle density of 100 mg/ml.

(v) HDA

MSPS (2.5g moist weight) is activated with oxirane groups as described for TAEA-MSPS, then suspended in 5M hexane-1,6-diamine (ml). The suspension is shaken at 30°C for 2 hours, then filtered, washed successively with sterile distilled water, 1M sodium chloride, sterile distilled water and finally 0.05M phosphate buffer (pH7). The HDA-MSPS is stored in 20% aqueous methanol to give a final particle density of 100 mg/ml.

(vi) EHEP

Cross-linked MSPS (0.9 g moist weight) is suspended in 40% sodium hydroxide (4ml) and cooled to 0°C in an ice-water bath. A solution of N-(2-hydroxyethyl)piperazine (0.5ml) in epichlorohydrin (0.9ml) is added drop-wise with occasional shaking, maintaining the temperature at 0°C. After 30 minutes the suspension is allowed to warm to 23°C, then shaken vigorously using a flask shaker and left to stand for 16 hours. The mixture is

then poured into a large volume of 1M hydrochloric acid with stirring, filtered, then washed successively with 1M sodium hydroxide, sterile distilled water, 20% aqueous methanol and finally sterile distilled water. The EHEP-MSPS is stored in 20% aqueous methanol to give a final particle density of 100 mg/ml.

Specific protocols for the separation and purification of different types of nucleic acids are given below.

Three specific protocols for the separation and purification of different types of nucleic acids have been developed:

(i) DNA isolation
(ii) Isolation of total RNA from nucleic acid mixtures
(iii) Isolation of plasmid DNA from a cell lysate mixture.

The nucleic acids to be adsorbed by the MSPS can be in a variety of forms, including aqueous solutions of one, or more than one, species of nucleic acid, or semi-crude cell lysate mixtures. Nucleic acids are adsorbed non-specifically by the MSPS at ambient temperature (23°C) from solution at pH7 or below. Nucleic acids can be eluted specifically in pure form from the MSPS by treatment with elution solutions of differing ionic strengths.

A mixture of RNA and DNA can be adsorbed simultaneously by the DEAE-derivatised beads, the RNA eluted using 0.1MNaCl/50mM arginine (free base) at 65°C for 30 minutes.

Plasmid DNA can be adsorbed by the MSPS from a cell lysis mixture (boiling lysis) which has been treated

with RNAse. Any remaining small fragments of oligoribonucleotide which co-adsorb with the plasmid DNA can be eluted using 0.1M/50mM arginine (free base), then the pure plasmid DNA can be eluted using 1.0M/50mM arginine (free base) at 65°C for 15 minutes.

Total RNA could be adsorbed to and eluted from the DEAE-MSPS, but elution occurred at much lower ionic concentrations and temperatures and at a significantly faster rate. DEAE-MSPS was incubated with a solution of total RNA (50μg ml^{-1}) at 23°C for 30 minutes, magnetically immobilised and the supernatant removed. Comparison of the A_{260} values of the supernatant and original stock solution of RNA showed that, on average, 90 % of the RNA had been adsorbed by the MSPS. The RNA could be eluted with an efficiency of 80-85% at salt concentrations of 0.1M NaCl up to 1.0M NaCl (50mM arginine free base) at 23°C.

Studies on the effect of elution solution composition on salmon sperm DNA elution showed that below 0.3M NaCl, negligible amounts of DNA were eluted, even at 65°C. Above 0.3M NaCl, DNA began to be eluted from the MSPS, and full elution was achieved at 1.0M NaCl/50mM arginine free base/65°C. The rate of DNA elution was much slower than that for RNA: samples of DEAE-MSPS which had adsorbed salmon sperm DNA were treated for 1, 5, 15 and 30 minute periods with elution solutions of 0.1, 0.5 and 1.0M NaCl (all 50mM arginine) at 65°C. No DNA was washed from the beads at 0.1M NaCl; at 0.5M NaCl, elution occurred but only up to a maximum of 68% of adsorbed DNA. Treating the particles with 1.0M NaCl led to 45% elution of DNA after 1 minute, but the full 30 minute period was still needed to achieve 90% elution (Table 2).

DEAE-MSPS can also be used for differentiating plasmid DNA and total cellular RNA. A solution of plasmid pUC 18 was prepared by standard boiling lysis, which contained amounts of RNA carried over from the lysis step. DEAE-MSPS was incubated with this solution under the same conditions used for other protocols, magnetically immobilised, then treated with 0.1M NaCl, to remove adsorbed oligoribonucleotides, then the plasmid DNA eluted using 1.0M/50mM arginine free base. Eluted samples were analysed by agarose gel electrophoresis, which showed firstly that the MSPS will adsorb and release plasmid DNA satisfactorily using the standard uptake and elution protocol, and secondly, that RNA was eluted at 0.05M-0.1 NaCl, whilst the plasmid DNA was eluted at a salt concentration of 1.0M NaCl. Therefore, DEAE-MSPS can be used to separate unwanted RNA resulting from cell lysis from plasmid DNA, and provide plasmid DNA of a purity and quality suitable for further applications.

Though exhibiting no preference in its adsorption of nucleic acids, DEAE-MSPS appears to selectively release them according to size upon application of elution solutions. The DEAE-MSPS was non-specific in its uptake of a mixtures of nucleic acids from solution, but by application of different concentrations of an elution solution, DNA and RNA could be eluted specifically from the DEAE-MSPS, a procedure which can be scaled down to a microtitre plate format without loss of efficiency. Similarly, plasmid DNA and RNA could be speciated on the DEAE-MSPS.

EXAMPLE 3 DNA isolation

25 mg (250µl of 100 mg ml^{-1} suspension) of DEAE-MSPS is aliquotted into an Eppendorf tube and washed twice with sterile distilled water. A crude cell lysate containing the DNA to be isolated (up to 50µg total) is added to the MSPS and the suspension mixed by end-over-end rotation at 23°C for 30 minutes. The MSPS is magnetically immobilised using a magnetic tube stand/holder and the supernatant removed. 1ml of an elution solution consisting of 1M NaCl/50mM arginine free base is added to the MSPS and the suspension mixed by end-over-end rotation at 65°C for 30 minutes. The MSPS is then magnetically immobilised and the supernatant containing the eluted DNA removed.

EXAMPLE 4

Isolation of total RNA from a nucleic acid mixture

Total RNA can be isolated from a mixture of RNA and DNA using a method similar to that described above. 250µl of a suspension of DEAE-MSPS (100 mg ml^{-1}) is aliquotted into an Eppendorf tube and washed twice with sterile distilled water. The nucleic acid solution containing RNA and DNA (up to 50µg total) is added to the MSPS and the suspension mixed by end-over-end rotation for 30 minutes at 23°C. The MSPS is magnetically immobilised and the supernatant removed, then 1ml of an elution solution (0.1M NaCl, 50mM arginine free base) is added. The suspension mixed by end-over-end rotation for 30 minutes at 65°C, the MSPS magnetically immobilised and the supernatant containing eluted RNA removed. A second elution procedure is then carried out using 1M NaCl/50mM arginine free base to elute DNA which remains adsorbed to the matrix.

EXAMPLE 5

Isolation of plasmid DNA from cell lysis mixture

1.5 ml of cell culture is spun in a microcentrifuge for 20 seconds in an Eppendorf tube. The supernatant is aspirated and the cell pellet resuspended in 350μl STET buffer (0.1M NaCl, 10mM Tris-HCl pH 8, 1m EDTA, 5% triton X-100) containing 2μl of RNAse A solution (10mg ml^{-1}). 25μl of a freshly prepared solution of lysozyme (10mg ml^{-1}) is added in 10mM tris-HCl, pH8. The solution is placed in a boiling water bath for 40 seconds, then spun in a centrifuge at 13,000 rpm for 10 minutes. The pelleted cell debris is removed using a disposable pipette tip, and the lysate solution added to 25mg of DEAE-MSPS (250μl of 100 mg ml^{-1} suspension) in an Eppendorf tube. The suspension is mixed by end-over-end rotation for 30 minutes at 23°C, the MSPS magnetically immobilised and the supernatant removed. The DEAE-MSPS is washed twice with sterile distilled water, and then washed two times with 0.1mM NaCl/50mM arginine free base to remove any oligoribonucleotides present in the lysis mixture which may become adsorbed to the MSPS. 1ml of elution solution (0.5M NaCl/50mM arginine free base) is then added and the suspension mixed by end-over-end rotation at 65°C for 15 minutes. The MSPS is magnetically immobilised and the supernatant containng purified plasmid DNA is removed. The DNA can then be precipitated, analysed directly by agarose gel electrophoresis, or manipulated in other ways.

TABLE 1

Efficiency of adsorbtion of salmon sperm DNA by differently derivatized MSPS

MSPS	Adsorbtion / %
DEAE	95
ECTEOLA	89
TAEA	95
Spermine	86
EHEP	67
HDA	40

25mg of MSPS incubated with 1ml of salmon sperm DNA (50µg ml^{-1}), 30min, 23°C.

(Average values of at least three experiments).

TABLE 2

Elution of adsorbed salmon sperm DNA from DEAE-MSPS using different elution solutions

[NaCl]	Elution / %			
	1min	5min	15min	30min
0.1	0	0	0	0
0.2	-	-	-	2
0.3	-	-	-	25
0.4	-	-	-	52
0.5	18	44	56	68
0.6	-	-	-	80
0.7	-	-	-	78
0.8	-	-	-	80
0.9	-	-	-	88
1.0	45	71	72	97

All elution solutions 50mM arginine free base. Elution for 30min, 65°C. 25mg of DEAE-MSPS incubated with 1ml of salmon sperm DNA (50µg ml^{-1}), 30min, 23°C. (Average values of at least three experiments).

CLAIMS

1. Magnetisable polymer-based particles derivatised with a ligand having direct binding affinity for nucleic acids or other types of biopolymer molecule.

2. Magnetisable particles according to claim 1, in which the polymer is in gel form.

3. Magnetisable particles according to claim 1 or 2, in which the polymer is cross-linked.

4. Magnetisable particles according to claim 2, or 3, in which the particles are beads.

5. Magnetisable particles according to any of claims 1 to 4 in which the polymer is agarose.

6. Magnetisable particles according to any of claims 1 to 5, in which the ligand is one capable of assuming a positive charge at pH7 or below and of reversibly binding directly to a negatively charged group or moiety in the target molecule.

7. Magnetisable particles according to any of claims 1 to 6, in which the ligand comprises a primary, secondary, or tertiary amine group.

8. Magnetisable particles according to claim 7, in which the ligand is selected from

(i) Diethylaminoethyl - DEAE
(ii) Epichlorohydrin/triethanolamine - ECTEOLA
(iii) Spermine
(iv) Diglycidylbutane-1,4,-diol/Tris(aminoethyl) amino - TAEA

(v) Epichlorohydrin/hydroxyethylpiperazine - EHEP

(vi) Diglycidylbutane-1,4,-diol/Hexane-1,6-diamino - HDA

9. Magnetisable particles according to any of the preceding claims, in which the magnetisable component comprises magnetic iron oxide.

10. Magnetisable particles according to any of the preceding claims having a particle size range of 20-150 μm.

11. Magnetisable particles according to any of the preceding claims, prepared by extrusion of an aqueous solution of the derivatised polymer containing suspended particles of a magnetisable material into a water-immiscible oil phase.

12. A method of preparing magnetisable particles according to any of the preceding claims, which comprises extruding a suspension of magnetisable particles in an aqueous solution of the derivatised polymer through an orifice into a continuously stirred water-immiscible oil phase.

13. A method according to claim 12, in which the orifice has a bore of 0.1 - 0.3 mms.

14. A method according to claim 12 or 13, in which the extrusion ratio is 2.0 - 3.0 ml/sec.

15. A method according to claim 12, 13 or 14, in which the oil phase is stirred with a rotary stirrer at 4000-6000 revolutions/min.

16. A method according to claims 12, 13 or 14 in which the oil phase is stirred by a homogeniser.

17. A method of separating biomolecules using magnetisable particles according to any of claims 1 to 11.

18. A method according to claim 17, for separating nucleic acids, from other biomolecules.

19. A method according to claim 17, for separating DNA or RNA from mixtures thereof or from other cell components.

20. A method according to claim 17, for separating plasmid DNA.

21. A method according to claim 18, 19, 20 in which the target substance is adsorbed on the magnetisable particles at pH 7 or below and eluted selectively with elution solutions of approprate ionic strength.

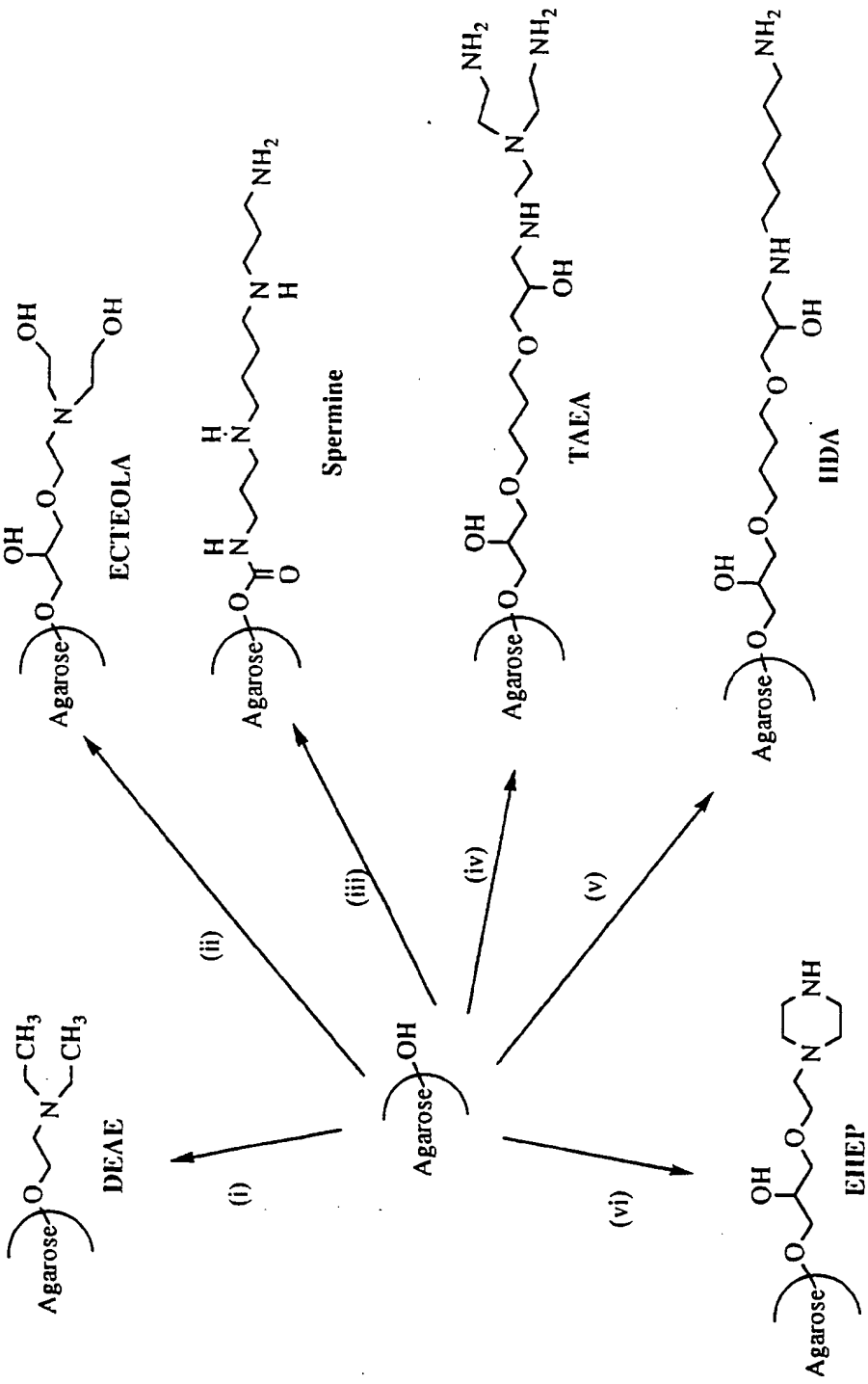

FIGURE 1

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 93/02289

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 B01J20/32 B01D15/08 G01N30/48 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 B01J B01D G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 335 094 (MOSBACH) 15 June 1982 see column 2, line 6 - column 4, line 11	1-5,9
A	EP,A,0 184 710 (BAYER) 18 June 1986 see page 4, line 23 - page 13, line 22 see page 17, line 11 - page 18, column 9	1,3,7,9, 11,12,17
A	US,A,4 452 773 (MOLDAY) 5 June 1984 see column 4, line 6 - column 5, line 28	1-7
A	WO,A,83 03426 (GAMBRO LUNDIA) 13 October 1983 see page 11-12; claims 1-9	1,5,11
A	WO,A,86 03136 (UNIVERSITY PATENTS) 5 June 1986	

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 February 1994	14. 02. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Wendling, J-P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/GB 93/02289

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 234 083 (TOSOH CORP.) 2 September 1987	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/GB 93/02289

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4335094	15-06-82	NONE		
EP-A-0184710	18-06-86	DE-A-	3444939	12-06-86
		JP-A-	61140523	27-06-86
US-A-4452773	05-06-84	CA-A-	1217934	17-02-87
WO-A-8303426	13-10-83	EP-A-	0116550	29-08-84
		JP-T-	59500399	15-03-84
		SE-A-	8201972	30-09-83
		US-A-	4687748	18-08-87
WO-A-8603136	05-06-86	EP-A-	0203163	03-12-86
		JP-T-	62501196	14-05-87
		US-A-	5167812	01-12-92
		US-A-	5167811	01-12-92
		US-A-	4675113	23-06-87
EP-A-0234083	02-09-87	JP-A-	62197425	01-09-87
		AU-B-	602489	18-10-90
		AU-A-	5981286	27-08-87
		DE-A-	3684905	21-05-92
		US-A-	5200270	06-04-93